Voltage-dependent Open-State Inactivation of Cardiac Sodium Channels: Gating Current Studies with Anthopleurin-A Toxin

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ABSTRACT The gating charge and voltage dependence of the open state to the inactivated state (O→I) transition was measured for the voltage-dependent mammalian cardiac Na channel. Using the site 3 toxin, Anthopleurin-A (Ap-A), which selectively modifies the O→I transition (see Hanck, D. A., and M. F. Sheets. 1995. Journal of General Physiology. 106:601–616), we studied Na channel gating currents (Ig) in voltage-clamped single canine cardiac Purkinje cells at ~12°C. Comparison of Ig recorded in response to step depolarizations before and after modification by Ap-A toxin showed that toxin-modified gating currents decayed faster and had decreased initial amplitudes. The predominate change in the charge-voltage (Q-V) relationship was a reduction in gating charge at positive potentials such that Qmax was reduced by 33%, and the difference between charge measured in Ap-A toxin and in control represented the gating charge associated with Na channels undergoing inactivation by O→I. By comparing the time course of channel activation (represented by the gating charge measured in Ap-A toxin) and gating charge associated with the O→I transition (difference between control and Ap-A charge), the influence of activation on the time course of inactivation could be accounted for and the inherent voltage dependence of the O→I transition determined. The O→I transition for cardiac Na channels had a valence of 0.75 e-. The total charge of the cardiac voltage-gated Na channel was estimated to be 5 e-. Because charge is concentrated near the opening transition for this isoform of the channel, the time constant of the O→I transition at 0 mV could also be estimated (0.53 ms, ~12°C). Prediction of the mean channel open time-voltage relationship based upon the magnitude and valence of the O→C and O→I rate constants from I_na and I_g data matched data previously reported from single Na channel studies in heart at the same temperature.

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J. Gen. Physiol. © The Rockefeller University Press · 0022-1295/95/10/617/24 $2.00

Volume 106 October 1995 617-640
INTRODUCTION

Ever since Hodgkin and Huxley (1952) proposed Na channel inactivation to have intrinsic voltage dependence, the magnitude of its voltage dependence has been uncertain. In 1977, Armstrong and Bezanilla (1977) proposed that inactivation of Na channels in squid giant axon had little or no voltage dependence based upon gating current (I_g) recordings of Na channels before and after internal exposure to the proteolytic enzyme, pronase. They proposed a model of inactivation as a particle or "ball" that would pivot on the end of a chain and bind to a receptor on the intracellular surface of the activated Na channel. In this model inactivation derived most of its apparent voltage dependence from coupling to voltage-dependent channel activation (Armstrong, 1981). Subsequently, single-channel experiments in neuroblastoma cells (Aldrich, Corey, and Stevens, 1983) demonstrated that inactivation was more rapid than activation and that decay of I_{Na} in response to step depolarizations represented in part declining numbers of channels that open for the first time. Additional studies of gating currents (Bullock and Schauf, 1979; Nonner, 1980; Vandenberg and Bezanilla, 1991a), of whole-cell I_{Na} recordings (Gonoi and Hille, 1987; Cota and Armstrong, 1989), and of single-channel recordings (Aldrich and Stevens, 1987; Vandenberg and Bezanilla, 1991a) have also concluded that inactivation of neuronal Na channels is largely voltage independent.

However, other Na channel gating current studies (Swenson, 1983; Stimers, Bezanilla, and Taylor, 1985; Greeff and Forster, 1991), single-channel studies (Horn, Vandenberg, and Lange, 1984; Vandenberg and Horn, 1984), and I_{Na} studies (Bean, 1981) in neuronal tissues have concluded that inactivation may be dependent upon voltage. In heart, voltage dependence of the O→I transition of Na channels is supported by single-channel studies that have shown mean channel open time to have a biphasic dependence upon voltage with the longest open times occurring near -40 mV (Berman, Camardo, Robinson, and Siegelbaum, 1989; Kirsch and Brown, 1989; Yue, Lawrence, and Marban, 1989; Scanley, Hanck, Clay, and Fozzard, 1990; Dudley and Baumgarten, 1993). From such studies, the valence of the O→I transition has been estimated to be 0.9 e- (Berman et al., 1989; Yue et al., 1989; Scanley et al., 1990). It has been suggested, however, that technical limitations of single-channel recordings make the shortening of mean open times at strongly depolarized potentials equivocal and that the conclusion of voltage dependence of the O→I transition may be unreliable (e.g., Patlak, 1991).

A component of I_g should have a time course similar to that for inactivation. Recently, we have developed an experimental preparation that permits measurement of cardiac Na channel gating currents in single canine cardiac Purkinje cells (Hanck, Sheets, and Fozzard, 1990). In this preparation we have shown that the site 3 toxin, Anthopleurin-A (Ap-A), predominantly modifies the O→I transition with minimal effect on Na channel activation and inactivation from closed states (Hanck and Sheets, 1995). Using Ap-A toxin we identified Na channel gating currents during step depolarizations that result from channels undergoing O→I transitions. The magnitude of this charge represents ~33% of the total gating charge recorded at positive potentials. Comparison of the gating charge resulting from Na channel activation to the gating charge associated with the O→I transition at test
potentials $\geq -20$ mV, the voltage dependence of the $O\rightarrow I$ transition was measured to be 0.75 $e^-$, and the time constant of the $O\rightarrow I$ transition at 0 mV was estimated to be 0.53 ms at 12°C. Our findings, which depend on a very different experimental paradigm from single-channel studies and which concentrate on a voltage range where single-channel measurements are difficult to obtain, confirm that inactivation of cardiac Na channels is voltage dependent. Some of these data have been presented in abstract form (Sheets and Hanck, 1993).

METHODS

Cell Preparation and Solutions

Cells were isolated from canine cardiac Purkinje fibers using the procedure previously described (Sheets et al., 1983; Hanck and Sheets, 1995). Canine Purkinje cells have been shown to have gating current signals that arise predominately from Na channels with very little contamination from other voltage-gated channels such as Ca channels under our experimental conditions (Hanck et al., 1990). For whole-cell $I_{Na}$ experiments, the control extracellular solution contained (millimolar) 15 Na+, 185 TMA+, 2 Ca2+, 4 Mg2+, 0.010 Cd2+, 200 MES-, 12 Cl-, and 10 HEPES (pH 7.2). For measurements of $I_{Na}$ was replaced with TMA+ and 10 µM saxitoxin (STX) (Calbiochem Corp., San Diego, CA) was added. We have previously found that block by STX could be reversed within 1-2 min of removal of STX from the bath (Hanck et al., 1990). Intracellular solution contained (millimolar) 200 TMA+, 200 F-, 10 EGTA, and 10 HEPES (pH 7.2). The increased osmolality of the solutions helped to decrease series resistance. Concentrations of Mg2+ and Cd2+ were chosen to maximize block of $I_{Ca}$ while producing minimum effects on $I_{Na}$ (Hanck and Sheets, 1992a; Sheets and Hanck, 1992). Temperature was controlled using a SensorTek (Physio Temp Instruments, Inc., Clifton, NJ) TS-4 feedback-controlled thermo electric stage placed adjacent to the bath chambers and typically varied $<0.5$°C during an experiment. Cells were studied between 10.5 and 12.5°C.

To study a near uniform population of Na channels a concentration of 340 nM Ap-A toxin was chosen for all studies because this concentration modified >90% of the Na channels and minimized the cost of experiments (Hanck and Sheets, 1995). Similar to previous reports (Neumcke, Schwarz, and Stampfl, 1985) we confirmed that Ap-A toxin was not displaced from cardiac Na channels by exposure to STX by demonstrating that Na channel inactivation remained modified by Ap-A toxin after exposure to a solution containing STX and no Ap-A toxin (data not shown).

Recording and Analysis

Recordings were made using a large bore, double-barrelled glass suction pipette for both voltage clamp and internal perfusion as previously described (Makielski, Sheets, Hanck, January, and Fozzard, 1987; Hanck and Sheets, 1993). To maximize the signal to noise ratio for gating current measurements protocols contained four repetitions at each test voltage that were .25 of a 60-Hz cycle out of phase, a procedure that helped in the rejection of 60 Hz noise. To determine the time course of the development of $I_{Na}$ inactivation a two-pulse development of inactivation protocol was used where the cell membrane (from a holding potential of $-150$ mV) was stepped to a conditioning potential ($V_c$) between $-40$ and $+60$ mV for variable durations before stepping back to $-120$ mV (or $-110$ mV) for 2 ms. Na channel inactivation was assayed from the ratio of peak $I_{Na}$ in a step to 0 mV relative to that in the absence of a conditioning pulse. The brief hyperpolarization to $-120$ mV (or $-110$ mV) for 2 ms was insufficient to allow for recovery from inactivation.

Data were capacity corrected using 16 scaled current responses to steps between $-150$ mV and $-190$ mV. Leak resistance ($R_L$) was taken as the reciprocal of the linear conductance between
−190 and −110 mV. For the 13 cells included in this study, \( R_L \) values were 119 ± 58 MΩ. Cell capacitance was measured from the integral taken over 10 ms of the current responses to voltage steps between −150 and −190 mV and was 90 ± 20 pF (\( n = 13 \) cells). Gating currents were leak corrected by the mean of the data between 6 and 10 ms for test potentials <0 mV or between 4 and 6 ms for test potentials ≥0 mV and integrated for 10 ms to determine gating charge. Running integrals rose to a stable plateau except, occasionally, when a small outward ionic current developed after a delay of several milliseconds at the most positive test potentials. Every other data point was deleted (resulting in 6.666 μs per data point) before fitting \( I_g \) relaxations by a sum of exponentials with DISCRETE (Provencher, 1976) to determine decay time constants, amplitudes, baselines, and standard errors of the estimate. This program provided a modified F-statistic that was used to evaluate the number of exponential components that best described the data.

Data were analyzed and graphed on a Masscomp 5450 computer using locally written programs (D. Hanck) or on a SUN Sparcstation (IPX or Sparc10/40 or 55) using SAS (Statistical Analysis System, Cary, NC). Unless otherwise specified, all summary statistics are expressed as means ± 1 SD. Regression parameters are reported as the estimate and the standard error of the estimate (SEE).

To account for spontaneous time-dependent changes in kinetic parameters during recordings the following procedure was used. Previously, we have shown that indices of cardiac Na channel kinetics (e.g., the half-point of peak conductance-voltage relationship) shift to more negative potentials as a linear function of time under our experimental conditions (Hanck and Sheets, 1992b). When indicated, kinetic indices were usually estimated by linear interpolation between measurements made in control and after washout of the toxin to account for any spontaneous background changes in channel kinetics.

**Na Channel Gating Current Simulation**

To help illustrate the effects of Ap-A toxin on Na channel gating currents, a simplified Markov kinetic model (Table I) representing Na channel behavior with and without a voltage-dependent \( O \rightarrow I \) transition was used to model gating current responses before and after inhibition of the \( O \rightarrow I \) transition. The addition of \( C \rightarrow I \) transitions with rate constants estimated from the time course of inactivation of \( k_{i0} \) from closed states (Hanck and Sheets, 1995) to the simplified Markov kinetic

<table>
<thead>
<tr>
<th>Transition</th>
<th>Rate Constant at 0 mV</th>
<th>Valency</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_1 )</td>
<td>5,500 ( s^{-1} )</td>
<td>0.50</td>
</tr>
<tr>
<td>( C_2 )</td>
<td>300 ( s^{-1} )</td>
<td>0.30</td>
</tr>
<tr>
<td>( C_3 )</td>
<td>7,000 ( s^{-1} )</td>
<td>0.50</td>
</tr>
<tr>
<td>( C_4 )</td>
<td>300 ( s^{-1} )</td>
<td>0.30</td>
</tr>
<tr>
<td>( C_5 )</td>
<td>2,500 ( s^{-1} )</td>
<td>0.90</td>
</tr>
<tr>
<td>( C_6 )</td>
<td>50 ( s^{-1} )</td>
<td>0.90</td>
</tr>
<tr>
<td>( O )</td>
<td>2,000* ( s^{-1} )</td>
<td>0.75†</td>
</tr>
<tr>
<td>( O \rightarrow I )</td>
<td>0.000004 ( s^{-1} )</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*AP-A toxin was simulated by slowing \( k_{i0} \) by a factor of 10−7.
†Voltage independence of \( O \rightarrow I \) was modeled by decreasing the valency of \( k_{i0} \) by a factor of 10−6.

\[ C_1 \leftrightarrow C_2 \leftrightarrow C_3 \leftrightarrow O \rightarrow I \]
Voltage-dependent Inactivation of Cardiac INa

model of the Na channel in Table I had minimal effects on gating currents over the voltage range considered in these experiments. Rate constants were assumed to be simple exponential functions of voltage,

\[ k(V) = k_{0mV} \times e^{qV_{m}/RT}, \]

where \( k_{0mV} \) is the rate constant at \( V_{m} = 0 \) mV, \( q \) is the voltage dependence of the rate constant, and \( e^{*} \) and \( F/RT \) have their usual meanings. Gating current was calculated as the product of the valence of the transition, the rate constant, and the probability of occupancy in the state appropriate for the transition. For example, the gating current resulting from channels associated with forward and backward \( O \rightarrow I \) transitions is as follows:

\[ I_{O \rightarrow I} = (q_{O} + q_{I}) \times [(k_{O \rightarrow I} \cdot P_{O}) - (k_{I \rightarrow O} \cdot P_{I})], \]

where \( q_{O} \) and \( q_{I} \) are the valencies associated with the \( O \rightarrow I \) and \( I \rightarrow O \) transitions, respectively, \( P_{O} \) is the probability of the channel occupying the open state, \( P_{I} \) is the probability of the channel occupying the inactivated state, and \( k_{O \rightarrow I} \) and \( k_{I \rightarrow O} \) are exponential functions of voltage as described above. It is important to recognize that the magnitude of \( I_{g} \) is dependent upon (a) the sum of the charge from both the forward and backward rate transitions, and (b) the probability that channels will proceed across the transition. For instance, even if the \( O \rightarrow I \) transition were voltage independent but the backward transition was voltage dependent, then gating current would be present as channels equilibrated between \( O \rightarrow I \).

The total gating current is the sum of the gating currents for each transition, and the total gating charge was calculated from the integral of the total gating current.

Table I shows the rate constants at 0 mV and their valencies used in the gating current simulations. Values for the transition \( k_{O \rightarrow I} \) were obtained from whole-cell measurements of \( I_{Na} \) (Hanck and Sheets, 1992b; Fig. 11), and those for \( k_{I \rightarrow O} \) were from the results reported in this paper. \( k_{0} \) was set to a very small value to make the inactivated state, \( I \), absorbing, and the valency was set equal to that for \( k_{0} \). The rate constants for closed-state transitions were estimated from Scanley et al. (1990) and their valency from estimates given in the Discussion. However, the determination of the valency of \( O \rightarrow I \) used in our analysis (see Fig. 10) was relatively insensitive to rate constants of closed-state transitions. To make \( k_{O \rightarrow I} \) voltage independent, its valency was decreased by a factor of \( 10^{-4} \). The effects of Ap-A toxin were simulated by slowing \( k_{O \rightarrow I} \) by a factor of \( 10^{-7} \). The simulations were performed on a Sun IPX Sparcstation running SAS using the Model procedure that solves multiple equations simultaneously by Newton’s method for nonlinear equations.

RESULTS

Gating Currents Are Reduced in Ap-A Modified Cardiac Na Channels

A family of gating currents and their integrals (charge) in control (A), after modification by Ap-A toxin (B), and after wash (C) are shown in Fig. 1. The magnitude of \( I_{g} \) in Ap-A toxin was reduced, and this is better appreciated by inspection of the corresponding integrals. As was the case for ionic current (Hanck and Sheets, 1995), the effects of Ap-A were reversed after washout of toxin. The decrease in gating charge did not result from an interaction between STX and Ap-A toxin because we observed similar reductions in gating charge in two cells in which \( I_{g} \) was recorded in the absence of STX by stepping to the reversal potential in symmetrical 15 mM Na-containing solutions (data not shown).

Similar changes after modification by Ap-A toxin were obtained in five other cells. The gating charge-voltage (Q-V) relationships for all six cells in control, after
FIGURE 1. Time course of $I_g$ (top) and charge (bottom) in control (A), after modification by 340 nM Ap-A toxin (B) and in wash (C). Step depolarizations to $-80, -55, -45, -30, -20, -10, 0, 10, 40$, and $60 \text{ mV}$ were made from a holding potential of $-150 \text{ mV}$. Note the difference in time scales between the top and bottom panels. Data shown are capacity and leak corrected, digitally filtered at $20 \text{ kHz}$, and with every sixth point plotted. The first $46 \text{ ms}$ were removed from the beginning of each sweep to eliminate the filtering artifact. Maximal charge decreased to $0.23 \text{ fC} \mu \text{m}^{-2}$ in Ap-A toxin from $0.34 \text{ fC} \mu \text{m}^{-2}$ in control. In wash maximal charge returned to $0.34 \text{ fC} \mu \text{m}^{-2}$. Cell L6.02: $C_m 140 \text{ pF}, R_L 160 \text{ M} \Omega$, $10.7^\circ \text{C}$.

modification by Ap-A toxin, and after washout of toxin are shown in Fig. 2. The most dramatic effect was the reduction in maximal gating charge ($Q_{\text{max}}$) by $33 \pm 1\%$. Similar to experiments measuring $I_{Na}$ (Hanck and Sheets, 1995) the effects of toxin were reversible; $Q_{\text{max}}$ returned to $94 \pm 0.07\%$ of the control value after washout of Ap-A toxin. The reduction in gating charge after Ap-A toxin occurred at test potentials $> -60 \text{ mV}$, while at more negative potentials there was a small increase in gating charge secondary to a shift in $V_{1/2}$. Similar decreases in charge were also observed in three cells with Cs$^+$ as the intracellular cation, indicating that the effect was not a result of a reduction in voltage-dependent block by TMA$^+$ (Hanck and Sheets, 1995). Before attributing the shift in $V_{1/2}$ to modification of Na channels by Ap-A toxin, it was necessary to account for the typical background “spontaneous” shift in kinetics that occurs as a function of time in this experimental preparation (see Methods). The mean rate of shift for six cells in control solutions was $-0.51 \pm 0.06 \text{ mV min}^{-1}$ and was similar to that we have previously reported (Hanck and Sheets, 1992b; Hanck, Makielski, and Sheets, 1994). The dotted line in Fig. 2 shows the control $Q-V$ relationship corrected for the delay in time between the gating charge measurement in control and in Ap-A toxin and illustrates that gating charge was reduced only at potentials positive to $-60 \text{ mV}$. After correction for the spontaneous shift the difference in $V_{1/2}$ in the presence of Ap-A toxin was $-6.2 \pm 2.3 \text{ mV}$. However, the change in $V_{1/2}$ did not result from a shift in activation gating charge of Na channels but rather reflected the fact that charge was selectively reduced only at positive potentials which causes an obligatory leftward shift.
Voltage-dependent Inactivation of Cardiac \( I_{Na} \)

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**Figure 2.** \( Q-V \) relationships for six cells in control (○), in 340 nM Ap-A toxin (●) and after wash (□). Means ± SEM are plotted. The solid lines represent the means of the parameters estimated from the fits to each data set of a Boltzmann distribution:

\[
Q = \frac{Q_{\text{max}}}{1 + e^{\frac{V - V_{1/2}}{k}}}
\]

where charge \( (Q) \) is that transferred during depolarizations to various voltages \( (V) \). Parameters estimated by the fit were maximum charge \( (Q_{\text{max}}) \), the voltage of the half point of the relationship \( (V_{1/2}) \), and the slope factor \( (k) \), expressed in millivolts. Parameters from the fits were as follows:

<table>
<thead>
<tr>
<th>Condition</th>
<th>( Q_{\text{max}} ) (( \mu \text{C/cm}^2 ))</th>
<th>( V_{1/2} ) (mV)</th>
<th>( k ) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37 ± 0.10</td>
<td>-49 ± 2</td>
<td>-12.6 ± 1.1</td>
</tr>
<tr>
<td>Ap-A toxin</td>
<td>0.25 ± 0.07</td>
<td>-61 ± 3</td>
<td>-12.9 ± 1.4</td>
</tr>
<tr>
<td>Wash</td>
<td>0.35 ± 0.08</td>
<td>-66 ± 7</td>
<td>-14.3 ± 1.6</td>
</tr>
</tbody>
</table>

The change in \( V_{1/2} \) between control and Ap-A toxin was \( -12.1 ± 3.6 \) mV, but this was largely accounted for by the background shift in kinetics and the fact that reduction in charge was concentrated at positive potentials. The dotted line is the control \( Q-V \) relationship corrected for the delay in time between measurements of the \( Q-V \) relationships between control and after Ap-A toxin (see text). The reduction in slope factor in wash was consistent with our previous observation that the slope factor of the \( Q-V \) relationship decreased late in cell life (Hanck et al., 1994).

The slope factors of the Boltzmann fits to the \( Q-V \) relationships were not significantly different between control and in Ap-A toxin. Thus, the predominate effect of Ap-A toxin on the \( Q-V \) relationship was a reduction in charge at more depolarized test potentials, the same potentials where \( I_{Na} \) inactivation was most rapid and nearly complete within 10 ms.

To determine whether the decrease in gating charge was associated with a change in the time course of \( I_g \), we analyzed \( I_g \) relaxations in control, in Ap-A toxin, and after wash. \( I_g \) were trimmed until the beginning of the decay phase (typically <100 \( \mu \)s), every other data point deleted, and relaxations fit with up to two exponential terms. We have previously shown under similar experimental conditions that the \( I_g \) relaxations were usually fit best with two exponential terms; the time constant contributing the greatest charge (dominant) had a biphasic dependence upon test potential, while the time constant contributing less to charge (minor) was generally faster (Hanck et al., 1990). Fig. 3 shows the results for six cells. After modification by Ap-A toxin the dominant tau \( (\tau_1) \) became faster particularly at test potentials \( > -60 \) mV (Fig. 3 A). Although the background shift in Na channel kinetics would be expected to produce faster decay of \( I_g \), that alone did not fully ac-
count for the decrease in $\tau_1$. If Ap-A toxin had not accelerated the decay of $I_g$, then the values in Ap-A toxin would have been intermediate between those in control and after washout of toxin. However, dominant $\tau$'s were typically superimposed upon those after washout. The faster decay of $I_g$ in Ap-A toxin was also associated with a reduction in the initial amplitude of $\tau_1$ (Fig. 3 B), and the initial amplitude returned to control values after washout.

To determine whether the changes in $\tau$'s and amplitudes were greater than those expected from the background shift in kinetics, the values of $\tau$'s and their initial amplitudes in control and after wash were averaged and then compared pairwise to those in Ap-A toxin for each voltage $>-60$ mV. The decreases in $\tau_1$ were significant at all test potentials with the exception of 0 and 10 mV (where the $p$ values were 0.18 and 0.06, respectively) and the decreases in initial amplitude were significant at all potentials ($p < 0.05$). The minor $\tau$'s ($\tau_2$) and their initial amplitudes in control were not significantly different from those in Ap-A toxin, although after washout of toxin (late in the cell's life) there appeared a small, rather long time constant at very positive potentials. Thus, the decrease in gating charge after modification by Ap-A toxin resulted from both a faster decay of $I_g$ relaxations and a decrease in initial amplitude.

The Difference Gating Charge between Control and Ap-A Toxin-modified Na Channels Represents Charge Associated with O--I Transitions

In the previous paper (Hanck and Sheets, 1995), we have shown that Ap-A toxin dramatically slowed the O--I transition with little or no effect on Na channel activation and inactivation from closed states (C--I). Furthermore, we have shown that
Ap-A toxin causes a reduction in gating charge at strongly depolarized test potentials where gating currents associated with Na channel inactivation should be most evident. These findings suggest that the difference between gating charge measured in Ap-A toxin and that in control may represent the gating charge associated with Na channels proceeding from the open to the inactivated state. To investigate this possibility, $I_g$ of Ap-A modified Na channels were subtracted from those measured in control and then compared to the time course of the development of $I_{Na}$ inactivation.

Fig. 4 A shows an example of the subtraction $I_g$ for one cell. At potentials $<-50$ mV no clearly recognizable signal could be resolved from the background noise. However, at a test potential of $-45$ mV there was a discernable subtraction $I_g$ signal which became obvious at more positive test potentials. The integrals of the subtraction $I_g$'s are shown in Fig. 4 B. At potentials between $-45$ and $-30$ mV, where many Na channels had not inactivated by 10 ms, the integrals did not reach a maximum. However, by $-20$ mV, the integrals reached a maximum as expected if nearly all channels had inactivated by 10 ms. The maximal charge measured from the integrals of the subtraction $I_g$ currents fully accounted for the difference in maximal charge. It is likely that at test potentials $<-45$ mV inactivation would be too slow and too small for gating currents associated with $O\rightarrow I$ to be accurately recorded under our experimental conditions. The mean of the subtraction $I_g$'s for six cells at $-20$ to 60 mV are shown in Fig. 4 C, and their corresponding integrals are shown in Fig. 4 D. The subtraction $I_g$'s were normalized to the maximum of the integral of each cell’s subtraction $I_g$’s before averaging. At a fast time scale a rising phase to the subtraction $I_g$’s is clearly noted as expected if channels first open before they inactivate (i.e., inactivation is coupled to activation). The subtraction $I_g$ at $-20$ mV peaked at $\sim0.9$ ms and shortened gradually as the test potential became more positive resulting in a faster rise time of the integrals (Fig. 4 D).
To illustrate that a rising phase should be present in subtraction $I_g$'s, we modeled the subtraction $I_g$'s resulting from channels undergoing $O\rightarrow I$ using the Markov kinetic model shown in Table I. Fig. 5 shows the results in the case when $O\rightarrow I$ is voltage dependent (A) and when it is voltage independent (B). Qualitatively, the subtraction $I_g$'s appear similar regardless of whether the $O\rightarrow I$ transition is voltage dependent or not, although the time-courses differ except at 0 mV where the $O\rightarrow I$ rate constants in both models are equal.

If the subtraction $I_g$'s represent the gating current resulting from Na channels traversing the $O\rightarrow I$ transition, then the time course of the subtraction gating charge at any given potential should correspond to the development of $I_{Na}$ inactivation at that same potential. To compare the time course between the integral of the subtraction $I_g$'s and the development of $I_{Na}$ inactivation, the subtraction gating charge from the cell in Fig. 4 was normalized to the maximal value (where inactivation should be complete). The time course of the development of $I_{Na}$ inactivation was measured using a two-pulse development of inactivation (see Methods) in four different cells at a similar temperature and time of internal perfusion. Fig. 6 shows the time course of the subtraction gating charge for potentials from -20 to 60 mV from the cell in Fig. 4 superimposed on the development of $I_{Na}$ inactivation average from four different cells. Note the nearly identical time courses as expected if subtraction charge resulted from Na channels transversing $O\rightarrow I$. Similar results were found for five other cells.

It is important to note that the time course of the charge integrals of subtraction $I_g$'s follow that of $I_{Na}$ inactivation regardless of whether the $O\rightarrow I$ transition is or is not voltage dependent. This is readily apparent by modeling the time course of the subtraction $I_g$'s in the Markov kinetic model with and without the $O\rightarrow I$ transition having voltage dependence, and comparing them to the time course of $I_{Na}$ inactivation. Fig. 7 shows the results for two potentials, -20 and +60 mV for the case where $O\rightarrow I$ is voltage dependent (A) or voltage independent (B). Note that the time courses are identical except at -20 mV where the normalized subtraction $I_g$ is similar but not identical to the development of $I_{Na}$ inactivation. The small difference at -20 mV results from a small number of open channels returning to closed states after the $O\rightarrow I$ transition is slowed.

To allow comparison of subtraction $I_g$ data to the development of $I_{Na}$ inactivation from all six cells, their time courses were fitted by a sum of exponentials and compared to one another (Fig. 8). Subtraction $I_g$'s were trimmed until they had reached a peak, every other data point deleted, and relaxations were fit with up to two exponentials. It should be noted that the number of points that were trimmed varied as a function of test potential (see Fig. 4). Relaxations were better fit with two exponential terms in 24 of 48 traces, but when detectable, the second exponential...
Figure 6. Comparison of normalized subtraction gating charge to the development of \(I_{Na}\) inactivation. For the two-pulse development protocol the duration of the conditioning pulse was varied from 0.3 to 24 ms, the membrane potential was briefly stepped to \(-120\) mV for 2 ms, and then stepped to 0 mV to measure peak \(I_{Na}\). The values of peak \(I_{Na}\) were normalized to that measured in the absence of a conditioning pulse. The smooth line represents the mean development of \(I_{Na}\) inactivation for four different cells at similar temperatures and durations of internal perfusion as those cells where gating currents were measured. The other line represents the gating charge obtained from subtraction of \(I_g\) in Ap-A toxin from that in control, integrated for 10 ms, and normalized to its maximum. The test potentials were \(-20\) mV (A), 0 mV (B), 20 mV (C), and 60 mV (D). Same cell as Fig. 4.

tial was usually very long with a low amplitude. Consequently, any second exponential was poorly resolved during the 10-ms time recording, and therefore time constants from single exponential fits were used for the comparison. Development of \(I_{Na}\) inactivation in four cells was measured using a two-pulse protocol as previously described. The time course of development of inactivation of \(I_{Na}\) was always best fit with two exponentials with a dominant, fast time constant and a second, slow time constant. Fig. 8 plots the dominant time constants of \(I_{Na}\) inactivation for test potentials between \(-40\) to 60 mV with the time constants of subtraction \(I_g's\) for the six cells and shows that the two measurements were nearly identical. It is important to note that the change in decay time constants does not represent the voltage dependence of the \(O\rightarrow I\) transition because the time course of the subtraction \(I_g\) also depends upon the time course of channel activation (i.e., channels must arrive in the open state before they inactivate).

In the model, the subtraction \(I_g's\) arise because the \(O\rightarrow I\) transition rate has been slowed such that channels do not undergo \(O\rightarrow I\) during 10 ms, and they become equal to the gating current resulting from channels traversing the \(O\rightarrow I\) transition at test potentials at which the probability of channels occupying the open state approaches one. However, in general in order for gating charge obtained from subtraction \(I_g's\) to represent the \(O\rightarrow I\) transition: (a) Ap-A toxin must modify the \(O\rightarrow I\) transition with little or no effect on channel activation. (b) After modification by Ap-A toxin channels should remain in the open state over much of the 10 ms recording and not redistribute between closed states. (c) Test potentials must be studied where the probability of Na channel transitions from \(G\rightarrow I\) does not change.
considered to be absorbing. Note that the time courses are identical except at -20 mV where the normalized subtraction $I_g$ is similar but not identical to the development of $I_{Na}$ inactivation. The small difference at -20 mV results from a small number of open channels (i.e., the steady state open-channel probability at -20 mV is 0.91) returning to closed states after the $O \rightarrow I$ transition is slowed.

after toxin modification, and it would be best if test potentials were used where there is a low probability of channels undergoing $C \rightarrow I$ either before or after modification by Ap-A toxin. (d) Channels that undergo $O \rightarrow I$ must move sufficient charge that the signal is brought out of the background noise, i.e., most channels must open then inactivate within 10 ms. All four conditions are present at test potentials $\geq -20$ mV under our experimental conditions. Evidence for points a-c are included in the accompanying paper (Hanck and Sheets, 1995), and we have shown here that at test potentials $\geq -45$ mV cardiac Na channels undergo $O \rightarrow I$ sufficiently fast that a subtraction gating signal can be measured (Fig. 4).

The Voltage Dependence of the $O \rightarrow I$ Transition Determined from the Relationship of Channel Activation to Channel Inactivation

To measure the voltage dependence of the $O \rightarrow I$ transition, the rate of channel activation must be taken into account. At positive potentials ($\geq -20$ mV) the gating
Voltage-dependent Inactivation of Cardiac $I_{Na}$

current in Ap-A toxin should represent only gating current associated with Na channel activation. Consequently, comparison of the time course of the normalized gating charge in Ap-A toxin, which represents channel activation, to the time course of normalized subtraction $I_g$'s, which represents channel inactivation from the open state, should permit the rate of channel activation to be taken into account, and therefore, the voltage dependence of the $O\rightarrow I$ transition to be measured.

Fig. 9 shows the results of the comparison of the time course of the normalized gating charge in Ap-A toxin to that of the normalized integral of subtraction $I_g$'s for one cell at test potentials of -20, 20, and 60 mV. At a test potential of -20 mV a large delay is apparent between Na channel activation and inactivation. However, at 60 mV the delay between the two traces is markedly less, and at 20 mV the delay is intermediate. If the time constant of the $O\rightarrow I$ transition were voltage independent, the delay between gating charge representing activation and the charge representing inactivation should be little changed as a function of potential, that is as channel activation becomes faster at more positive potentials, channels arrive in the open state sooner and consequently inactivate sooner but with a similar delay. Such a relationship between activation and inactivation gating charge becomes readily apparent from the model using the kinetic parameters in Table I. Fig. 10 shows the relationship between the normalized gating charge after “Ap-A toxin simulation” and the normalized integral of subtraction $I_g$'s when $O\rightarrow I$ is voltage dependent (left) or voltage independent (right). In addition, the normalized gating

![Normalized Na channel gating charge measured in Ap-A toxin (leftmost trace in each panel) and normalized subtraction Na channel gating charge (rightmost trace in each panel) for one cell. The test potentials were -20 mV (A), 20 mV (B), and 60 mV (C). The decrease in the delay between the two traces as a function of test potential results from the voltage dependence of the $O\rightarrow I$ transition (see text). The delay between the two traces was measured at a normalized charge of 0.6 and is represented by the horizontal solid line. The delay decreased from 1.2 ms at -20 mV to 0.12 ms at 60 mV. (D) Semilogarithmic plot of the means and SEM of the delay between the two traces for six cells as a function of test potential. The line represents the mean of the parameters calculated from least-squares fits to each dataset ($n = 6$). The line has a slope of $-0.051 \pm 0.006$ ln unit mV$^{-1}$, which corresponds to a valence of $0.75 \pm 0.15 e^-$, and an intercept at 0 mV of $-0.63 \pm 0.38$ ln units, which corresponds to a $\tau_{O\rightarrow I, 0\ mV}$ of $0.53 \pm 0.20$ ms. Cell L6.03; $C_m 87$ pF, $R_l 210$ M$\Omega$, 10.8°C.]
FIGURE 10. Model simulations (using parameters in Table I) of the relationships of normalized activation gating charge after "Ap-A toxin modification" to normalized subtraction \( I_g \)'s when \( O \rightarrow I \) is voltage dependent (A) or voltage independent (B). The leftward solid line in each panel represents the activation gating charge, the rightward solid line represents the gating charge of subtraction \( I_g \)'s. Also shown is the normalized gating charge associated when channels traverse the \( C_i \rightarrow O \) transition (dotted line). The bottom panel in each column plots the In of the delay between the activation gating charge and the charge of subtraction \( I_g \) when they both have a value of 0.6 as a function of test potential (solid line) and the corresponding In of the delay between the normalized gating charge associated with the \( C_i \rightarrow O \) transition and that of the subtraction \( I_g \)'s (dotted line). The slopes and intercepts were as follows:
charge of channels traversing the $C_s \rightarrow O$ transition (dotted line) is also shown. When $O \rightarrow I$ is voltage dependent the delay between the normalized gating charge associated with activation and that associated with $O \rightarrow I$ becomes shorter throughout the voltage range. However, when the $O \rightarrow I$ transition is voltage independent the delay remains almost constant throughout the voltage range. At very positive potentials activation would become almost "instantaneous," the time course of $O \rightarrow I$ would approximate a single exponential, and the delay between activation and inactivation would represent the time constant of the $O \rightarrow I$ transition when the fraction of the normalized integral of subtraction $I^+_n$'s equalled 0.63.

However, in the case when the $O \rightarrow I$ transition is voltage dependent the delay between the normalized activation gating charge and normalized inactivation gating charge decreases exponentially as a function of potential; the slope of the log transform of the delay gives the voltage dependence of the $O \rightarrow I$ transition (Fig. 10, left). In the model with $O \rightarrow I$ having a voltage dependence of 0.75 $e^-$ and a $\tau_{O \rightarrow I, 0 \text{ mV}}$ of 0.50 ms, the slope of the line is $-0.091 \text{ ln unit mV}^{-1}$ (which corresponds to a valency of 0.75 $e^-$) and an intercept of 0.74 ms at 0 mV when the delay is measured at 0.6 (Fig. 10, left). The valence of the $O \rightarrow I$ transition is relatively insensitive to the value at which the delay is measured because the inactivation gating charge is coupled to the activation gating charge. For example, if the delay is measured using a normalized value of 0.3 instead of 0.6, the log of the delay as a function of potential is still well fit by a line with a slope that gives a valency of 0.77 $e^-$ (data not shown). However, measurement of the delay when the normalized charge approximates 0.63 should give the fitted line an intercept which estimates the time constant of the $O \rightarrow I$ transition. When the delay between the normalized gating charge associated with the $C_s \rightarrow O$ transition and the inactivation gating charge is measured at a value of 0.60, the slope remains unchanged but the intercept gives a better estimate of $\tau_{O \rightarrow I, 0 \text{ mV}}$ (0.50 ms, which is the same value as the model). Thus, whenever a greater fraction of the activation charge is associated with the $C_s \rightarrow O$ transition, which has been shown to be the case for cardiac Na channels (Hanck et al., 1990),

<table>
<thead>
<tr>
<th>Voltage-dependent $O \rightarrow I$ inactivation with model parameters of 0.75 $e^-$ valency and $\tau_{O \rightarrow I, 0 \text{ mV}}$ of 0.50 ms</th>
<th>Voltage-independent $O \rightarrow I$ inactivation with model parameters of $\tau$ constant at 0.50 ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delay using activation charge</td>
<td>Delay using charge of $C_s \rightarrow O$ transition</td>
</tr>
<tr>
<td>Intercept (ms)</td>
<td>0.74</td>
</tr>
<tr>
<td>Valency ($e^-$)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

The slight deviation from a slope of 0 for the model with no voltage dependence of the $O \rightarrow I$ transition occurs because the steady state channel open probability after "Ap-A toxin" is slightly $< 1$ at the more negative potentials (see text).
and when the forward rate constant of the $G_{C\rightarrow O}$ transition becomes faster, the normalized activation charge better approximates the normalized gating charge associated with the $G_{C\rightarrow O}$ transition. In such instances the intercept will be a more accurate estimate of $\tau_{O\rightarrow I, 0 \text{mV}}$.

For these reasons, we selected to measure the delay at a fraction of 0.6 of normalized charge over a potential range from $-20$ to $+60$ mV (Fig. 9). The delays between the two charge traces were well described by an exponential with a slope of $-0.031 \pm 0.006$ ln units mV$^{-1}$ and an intercept at 0 mV of $-0.63 \pm 0.38$ ln units. The slope gives a voltage dependence of the $O\rightarrow I$ transition of 0.75 $e^-$, and the intercept gives an estimate of $\tau_{O\rightarrow I, 0 \text{mV}}$ at 0 mV of 0.53 $\pm$ 0.20 ms.

**DISCUSSION**

We have isolated gating currents associated with $O\rightarrow I$ in cardiac Na channels by subtracting gating current records recorded in the presence of Ap-A toxin from gating currents measured in control. The integrals of the subtraction $I_g$'s represent the direct measurement of gating charge of channels traversing the $O\rightarrow I$ transition and contribute ~33% to the total Na channel gating charge. By comparison of the gating charge associated with Na channel activation to that associated with $O\rightarrow I$ we have shown the $O\rightarrow I$ transition to be voltage dependent with a valence of 0.75 $e^-$ and to have an estimated time constant at 0 mV of 0.53 ms at $\approx 12^\circ C$.

**Figure 11.** Time constants of $O\rightarrow C$ and $O\rightarrow I$ transitions calculated from $I_{so}$ and $I_g$ in cardiac Purkinje cells and mean Na channel open times measured from patch clamp recordings of single cardiac Na channels and calculated from the time constants for $O\rightarrow C$ and $O\rightarrow I$ transitions. (A) $\tau_{O\rightarrow C}$ (solid line) and $\tau_{O\rightarrow I}$ (dotted line) were calculated from the equation:

$$\tau = \tau_0 \cdot e^{-V_m/24.5}$$

where $\tau$ is the time constant at each test potential, $\tau_0$ is the time constant at 0 mV, $z$ is the valence of the state transition, $V_m$ is the membrane potential, and 24.5 is $kT/\varepsilon$ at $12^\circ C$. Values for $\tau_{O\rightarrow I}$ are from the text and those for $\tau_{O\rightarrow C}$ are from Hanck and Sheets (1992b) with a valence of 0.9 $e^-$ and a time constant of 0.20 ms at $-120$ mV for whole-cell voltage-clamped single Purkinje cells at $12^\circ C$. (B) Mean open times (line) calculated from time constants in A where $\tau_{\text{open}} = 1/(\tau_{O\rightarrow C}^{-1} + \tau_{O\rightarrow I}^{-1})$. Mean Na channel open times from Scanley et al. (1990) represents the recordings from three patches of canine Purkinje cells at $13^\circ C$, data from S. Hirai (personal communication) represents the mean from seven patches of guinea pig ventricular cells at $12^\circ C$, and data from Dudley and Baumgarten (1993) represents the mean of 18 patches of rabbit ventricular cells at $10^\circ C$. The slightly longer mean open times from Dudley and Baumgarten (1993) most likely result from the cooler temperature of $10^\circ C$. 
The time constants of the $O \rightarrow I$ transition as a function of potential calculated from our measurements of valence and time constant at 0 mV are plotted in Fig. 11A. Also shown are the time constants of the $O \rightarrow C$ transition determined from whole-cell voltage-clamp recordings of $I_{Na}$ under similar experimental conditions (Hanck and Sheets, 1992b). From these two relationships Na channel mean open times were predicted. The predictions, which depended on completely different experimental paradigms than do single channel studies, matched the mean open times measured from patch clamp studies of single cardiac Na channels at $\approx 12^\circ C$ (Fig. 11B). This consistency between data obtained using different experimental methods provides additional support for the conclusion that the $O \rightarrow I$ transition has significant voltage dependence in cardiac Na channels.

**Potential Errors in the Measurement of the $O \rightarrow I$ Transition**

To investigate the voltage dependence of the $O \rightarrow I$ transition we chose a method of analysis that relies on a minimum of assumptions of the kinetic model for the Na channel. We used test potentials ≥−20 mV to minimize the contributions of Na channels undergoing $C \rightarrow I$ transitions. Pathways other than channels undergoing $O \rightarrow I$ or remaining open during the 10-ms step depolarization. Although the measurement of the valency of the $O \rightarrow I$ transition is largely independent of the number closed, rested states before channel opening, the estimation of its time constant of the $O \rightarrow I$ transition can be affected as described previously.

A possible error may be introduced by any slowing of Na channel activation after Na channel modification by Ap-A toxin (Hanck and Sheets, 1995). Slowing of activation could arise from either a change in its valency or a change in the rate constants. The slope factor of the $Q-V$ relationship before and after Ap-A toxin was unchanged, which suggests that the overall voltage dependence of activation is unchanged. If rate constants during activation were slowed by Ap-A toxin, then $I_g$ relaxations after Ap-A toxin should also be slowed, and subtraction $I_g$'s would have a slightly faster rise time. Similarly, if activation rate constants were slowed by Ap-A toxin the time to peak of the subtraction $I_g$'s would occur sooner than the time to peak of unmodified $I_{Na}$. In addition, the delay between normalized activation gating charge and normalized integrals of subtraction $I_g$'s would be shortened. However, the change in delay as a function of voltage, which measures the valency of the $O \rightarrow I$ transition, should remain unchanged because the error resulting from the slowing of activation would be contained in both measurements and would factor out. For example, in the model shown in Fig. 10 with the $O \rightarrow I$ transition having a $\tau_{O \rightarrow I}$ of 0.50 ms and a valency of 0.75 e$, slowing of activation during "Ap-A toxin" was simulated by decreasing the forward rate constant of the $C \rightarrow O$ transition by one third from 2,500 s$^{-1}$ to 1,650 s$^{-1}$. In this case the time delay between the activation gating charge and the charge of subtraction $I_g$ at value of 0.6 as a function of test potential gave the same valency of 0.75 e$ but the $\tau_{O \rightarrow I}$ at 0 mV was underestimated (0.47 ms instead of 0.74 ms, Fig. 10). In the same model with slowed activation, the time to peak of the subtraction $I_g$'s was shortened by about a third compared to the time to peak of $I_{Na}$. The same effect may explain the shorter time to peak of the subtraction $I_g$'s (Fig. 4) compared to the time to peak $I_{Na}$ (Hanck and Sheets, 1992b, 1995).
Although we have demonstrated that the $O\rightarrow I$ transition is voltage dependent, the reduction in total gating charge by 33% after removal of a transition accounting for 0.75 $e^-$ may appear excessive. However, the magnitude of the gating charge associated with channels traversing the $O\rightarrow I$ transitions represents the product of the number of channels making the transition times the total gating charge associated with the transition, which is the sum of the valence of the $O\rightarrow I$ transition and that of the $I\rightarrow O$ transition. Thus, the valence of the $O\rightarrow I$ transition need not account for all of the reduction in charge after modification by Ap-A toxin if the $I\rightarrow O$ transition were also voltage dependent (Patlak, 1991). It is generally agreed that recovery from inactivation is voltage dependent. For Na channels in squid giant axon Vandenberg and Bezanilla (1991b) estimated the voltage dependence of $I\rightarrow O$ to be 0.9 $e^-$, and recently, Kuo and Bean (1994) have been able to demonstrate that a small fraction of Na channels in rat hippocampal CA1 neurons undergo $I\rightarrow O$ transitions during recovery from inactivation. If we assume the voltage dependence of the $I\rightarrow O$ transition of Na channels in heart to be the same as that in squid, then the total gating charge associated with channels traversing the $O\rightarrow I$ transition would be approximately $1.7 \times (0.75 + 0.9 \times e^-)$, or, if symmetrical energy barriers were assumed the total would be $1.5 \times e^-$. Because the gating charge associated with the $O\rightarrow I$ transition represents approximately one third of the total gating charge (see Fig. 2), the total gating charge for a cardiac channel is predicted to be almost $5 \times e^-$. The estimate of 5 $e^-$ per channel is similar to values previously predicted from the relationship of $I_{Na}$ density to $Q_{max}$ for cardiac cells (Hanck et al., 1990) and to values predicted for Na channels in other tissues (see Hille, 1992). However, it has been recently reported that heterologous expressed skeletal muscle Na channels may have as many as 10–11 $e^-$ per channel (Patlak, Rovner, Lieberman, and Hirschberg, 1995), which would approach the 12–16 $e^-$ recently reported for voltage-gated K channels (Schoppa, McMormack, Tanouye, and Sigworth, 1992; Perozo, Mackinnon, Bezanilla, and Stefani, 1993; Zagotta, Hoshi, Dittman, and Aldrich, 1994).

Additionally, the gating charge associated with $C\leftrightharpoons C$ transitions in heart can be estimated. From single Na channel studies (Yue et al., 1989; Scanley et al., 1990) and $I_{Na}$ tail-current measurements (Hanck and Sheets, 1992b), the valence of $C\rightarrow O$ is estimated to be $\sim 1.8 \times e^- + 0.9 \times e^-$. Thus, if $O\rightarrow I$ contributed 1.7 $e^-$ and if total gating charge were $5 \times e^-$, then the valence for all of the $C\leftrightharpoons C$ transitions would be $\sim 1.5 \times e^-$. This prediction of a rather modest contribution to voltage dependence of activation by $C\leftrightharpoons C$ transitions is consistent with our previous finding that the mid-points of the peak conductance-voltage plot and the $Q-V$ relationship are well matched, suggesting a major portion of the charge is associated with transitions close to channel opening for this channel isoform (Hanck et al., 1990).

Our data suggest that after Na channel modification by Ap-A toxin the $Q-V$ relationship reflects only that gating charge associated with channel activation. Even in control solutions gating charge measured at $I_{Na}$ threshold potentials is likely to reflect only charge associated with activation of the Na channel, because both gating
Voltage-dependent Inactivation of Cardiac \( I_{\text{Na}} \) currents from \( O \rightarrow I \) and \( C \rightarrow I \) transitions are probably too small and too slow to be accurately measured. If all the gating charge could be measured during step depolarizations near \( I_{\text{Na}} \) threshold, the base of the \( Q-V \) relationship would be expected to show more gating charge than we have measured. The apparent voltage dependence of channel gating, as estimated by the slope factor of the Boltzmann fit to the \( Q-V \) data after \( \alpha_{p-A} \) toxin (see Fig. 2) was essentially unchanged (\(-12.6 \text{ mV vs } -12.9 \text{ mV}\)), which gives a valency of \(~2 \epsilon^+\). This is less than the \( 3-3.5 \epsilon^+ \) previously predicted for all of channel activation. It should be noted, however, that this is not unexpected because the total valence of gating in multistate systems is underestimated from the steepness of the \( Q-V \) relationship (Almers, 1978; Armstrong, 1981).

Previous Studies of Na Channel Inactivation

In one of the first comprehensive gating current studies of Na channels in giant squid axon, Armstrong and Bezanilla (1977) proposed that inactivation was voltage independent with the apparent voltage dependency resulting from coupling of inactivation to activation. They could not identify any ON-gating currents resulting directly from inactivation of Na channels, although they demonstrated that the time course of inactivation of \( I_{\text{Na}} \) was similar to the time course of "loss" of gating charge (gating charge immobilization) and that recovery of immobilized gating charge followed the time course of recovery from \( I_{\text{Na}} \) inactivation. Similar findings were reported by Meves and Vogel (1977). Subsequently, charge immobilization associated with \( I_{\text{Na}} \) inactivation has been well documented in frog myelinated nerve (Nonner, 1980), crayfish giant axons (Starkus, Fellmeth, and Raynor, 1981) and in Myxicola giant axons (Bullock and Schaaf, 1979). In addition, many studies of single Na channel currents in noncardiac tissue have modeled the \( O \rightarrow I \) transition to have a valence of only a few tenths of an \( \epsilon^+ \) (Aldrich et al., 1983; Aldrich and Stevens, 1987; Vandenberg and Bezanilla, 1991a). In whole-cell \( I_{\text{Na}} \) studies in which inactivation was removed (Gonoi and Hille, 1987; Cota and Armstrong, 1989) the \( G_{\text{Na}}-V \) relationship shifted ~\(-8 \text{ to } -20 \text{ mV} \) (depending on the agent), consistent with a rapid, essentially voltage-independent \( O \rightarrow I \) transition.

However, other studies in neuronal and skeletal muscle preparations have concluded that inactivation may be voltage dependent. In crayfish giant axon Bean (1981) suggested that the \( O \rightarrow I \) transition is voltage dependent based upon measurements of \( I_{\text{Na}} \). Swenson (1983) observed a slow component of \( I_{\text{g}} \) in crayfish giant axon that had time constant comparable to the time constant of decay of \( I_{\text{Na}} \), and suggested that inactivation is voltage dependent. Stimers et al. (Stimers et al., 1985) compared the relationship of the normalized peak conductance-voltage curve to the \( Q-V \) relationship in squid giant axon before and after treatment with pronase, and concluded that a kinetic model with the \( O \rightarrow I \) transition having a voltage dependence of \( 0.6 \epsilon^+ \) was necessary to fit their data. Greeff and Forster (1991) constructed isochronal plots of amplitudes of \( I_{\text{g}} \) vs the time derivative of the \( I_{\text{Na}} \) conductance in squid giant axon and calculated a total charge associated with the transition between the open state and inactivated state of \( 1.2 \epsilon^+ \) and estimated the voltage dependence of \( O \rightarrow I \) to be \(~0.52 \epsilon^+\). Furthermore, single-channel studies in GH3 cells have suggested a voltage dependence of \( O \rightarrow I \) between 0.7 to 1.9 \( \epsilon^+ \) (Horn et al.,
1984; Vandenberg and Horn, 1984). By quantifying the depolarization-induced reversal of α-scorpion toxin action from voltage-clamped $I_{Na}$ in toad myelinated nerve Strichartz and Wang (1986) estimated inactivation to be voltage dependent with a valency close to 1 $e^-$. The ~40% reduction in total gating charge of Na channels from frog myelinated nerve after modification by either crude Leiurus venom (Nonner, 1979) or by ATX II toxin (Neumcke et al., 1985) could be explained by the voltage dependence of Na channel inactivation. The recent work by Chahine, George, Zhou, Ji, Sun, Barchi, and Horn (1994) demonstrating the contribution of a charged amino acid in the S4 segment of domain IV in the human skeletal muscle Na channel to the rate of inactivation from the open state provides additional evidence for the voltage dependence of inactivation. Part of the variation in the previous studies may result from differences in the magnitude of the voltage dependence of $O\rightarrow I$ inactivation between isoforms of the Na channel just as different isoforms have various rates of channel activation.

**Model Implications**

Based on mutagenesis studies, a view of the Na channel's structure-function relationship is emerging. The voltage-dependent behavior of voltage-gated channels has been proposed to be closely associated with charged amino acid residues in the S4 repeats (Stühmer, Conti, Suzuki, Wang, Noda, Yahagi, Kubo, and Numa, 1989; Krafte, Goldin, Auld, Dunn, Davidson, and Lester, 1990). Strong evidence suggests that the intracellular linker region between domains III and IV comprises the inactivation gate (see review by Goldin, 1993). We suggest that the S4 segment of domain IV comprises the voltage sensor associated with rapid channel inactivation. In addition to the data reported here, additional support for involvement of the domain IV in rapid channel inactivation comes from studies of α-scorpion toxin, another site 3 toxin, showing that it binds to the extracellular loops between the S5 and S6 segments of both domains I and IV (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989). However, perhaps the strongest evidence for involvement of the S4 repeat in domain IV in rapid channel inactivation comes from studies of the human skeletal muscle sodium channel mutation found in paramyotonia congenita where either a cysteine or histidine is substituted for an arginine at position 1448 in the S4 of domain IV (Ptacek et al., 1992). Single-channel studies of those mutations are consistent with a delay in inactivation from the open state with little or no effect on C→I transitions or on channel activation (Chahine et al., 1994).

A Markov model for the cardiac Na channel, which has similarities to previous suggested models (Keynes, 1991; Patlak, 1991) and which incorporates many of these features is as follows:

\[
\begin{align*}
C_1 & \leftrightarrow (S_1, S_2) & (S_7, S_3) & (S_7, S_3, S_4) \\
C_2 & \leftrightarrow C_4 & O & O^* \\
I_1 & \leftrightarrow I_2 & I_3 & I_4 \\
(S_7, L) & \leftrightarrow (S_7, S_9, L) & (S_7, S_9, L) & (S_7, S_9, L)
\end{align*}
\]
S4 segments of domains I-III are associated with channel activation while the S4 of domain IV is associated with the $O \rightarrow I$ transition. When the S4 segments of domains I-III move the channel is open. After the channel has opened the S4 segment of domain IV moves, resulting in a second brief open state, $O^*$, and causing the inactivation gate (L) to bind rapidly to its receptor. Under these conditions the maximal amount of gating charge would be moved. The affinity of the receptor for the inactivation gate would be greatest after movement of all four S4 segments, and the inactivation gate would remain bound until repolarization of the membrane potential. Such a high affinity would allow the inactivated state $I$, to be an absorbing state. In addition, high affinity binding of the inactivation gate to its receptor would be manifest as slow recovery of gating charge during hyperpolarization, i.e. “charge immobilization.” Support for this model also comes from a recent paper in which a synthetic inactivation peptide was shown to “restore” inactivation in “gate deficient” Na channels or when inactivation was modified with a site 3 toxin (Eaholtz, Scheuer, and Catterall, 1994).

Na channel inactivation directly from closed states (Bean, 1981; Horn, Patlak, and Stevens, 1981; Lawrence, Yue, Rose, and Marban, 1991) could be accounted for by partial activation, i.e., movement of one or two of the three activation S4 segments, that would allow the inactivation gate (L) to bind to its receptor but with lower affinity and without the channel opening. In such a model $C \rightarrow I$ transitions have no intrinsic voltage dependence but receive apparent voltage dependence from coupling to channel activation. The inherent difference in the pathways between $C \rightarrow I$ and $O \rightarrow I$ may be involved in the fractionation of gating charge into immobilizable and nonimmobilizable components that has led to the concept of parallel gating particles associated with inactivation (Greeff, Keynes, and Van Helden, 1982; Keynes, Greef, and Van Helden, 1982; Bekkers, Forster, and Greef, 1990; Starkus and Rayner, 1991).

Although the binding of the inactivation gate after movement of the S4 of domain IV is rapid, our data cannot distinguish whether the binding is instantaneous or occurs after a few hundreds of microseconds. Figs. 6 and 8 suggest that the time course of the integral of subtraction $I_g$'s may be slightly more rapid than the time course of inactivation of $I_{Na}$ and implies that the gating current associated with inactivation may precede the binding of the inactivation gate. However, our findings may also be explained by the slight slowing of activation by Ap-A toxin (see above, Potential Errors in the Measurement of the $O \rightarrow I$ Transition). Nevertheless, if the movement of the S4 in Domain IV and the binding of the inactivation gate were not instantaneous but were separated by up to hundreds of microseconds, it may help to explain differences between isoforms of Na channels. Mean Na channel open times would be a function of not only the voltage range and rate constant of movement of the S4 of Domain IV but also the delay in binding of the inactivation linker to its receptor.

One proposed mechanism of action of site 3 toxins is that the toxin may physically inhibit movement of the S4 segment of domain IV after binding to channel’s extracellular loops. Because the inactivation gate has not been modified it could still bind to its receptor by $C \rightarrow I$ pathways. One explanation for the more rapid unbinding of site 3 toxins from Na channels during maintained depolarization (for
review, see Norton, 1991) may be that the energy from the electrical field can overcome the binding energy of the toxin/channel complex. Although this model does not account for slow (or ultraslow) inactivation of voltage-gated ion channels, it does account for many of the experimental observations concerning fast Na channel inactivation.

We thank Stephanie Krueger at Northwestern University Medical School for her excellent technical assistance. This study was supported by National Heart, Lung and Blood Institute Grants HL-R29-44630 (M. F. Sheets) and HL-P01-20592 (D. A. Hanck). D. A. Hanck is an Established Investigator of the American Heart Association.

Supported by NIH Grant HL-R29-44630 to M. F. Sheets and HL-P01-20592 to D. A. Hanck.

Original version received 25 April 1994 and accepted version received 3 April 1995.

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